THE CHARACTERIZATION OF THE ASPARTATE TRANSCARBAMOYLASE THAT IS FOUND IN THE *PYRBC'* COMPLEX OF *Bordetella pertussis*

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An aspartate transcarbamoylase (ATCase) gene from *Bordetella pertussis* was amplified by PCR and ligated into pT-ADV for expression in *Escherichia coli*. This particular ATCase (*pyrB*) was an inactive gene found adjacent to an inactive dihydroorotase (DHOase) gene (*pyrC'*). This experiment was undertaken to determine whether this *pyrB* gene was capable of expression alone or if it was capable of expression only when cotransformed with a functional *pyrC'*. When transformed into *E. coli* TB2 *pyrB*$^{-}$, the gene did not produce any ATCase activity. The gene was then co-transformed into *E. coli* TB2 *pyrB*$^{-}$ along with a plasmid containing the *pyrC'* gene from *Pseudomonas aeruginosa* and assayed for ATCase activity. Negative results were again recorded.

ACKNOWLEDGMENTS

I would like to respectfully acknowledge those, without whom, this thesis would not have been possible. They include my committee, Drs. Mark Farinha, Gerard O'Donovan, and Robert Benjamin, and my fellow graduate students who helped me beyond what might have been expected of them, Chris Fields, Alan Kumar, Andy Meixner, Dana Brichta, and Emily Carey. Thank you all for your assistance, advice, and support.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF ILLUSTRATIONS

Figure Page

INTRODUCTION

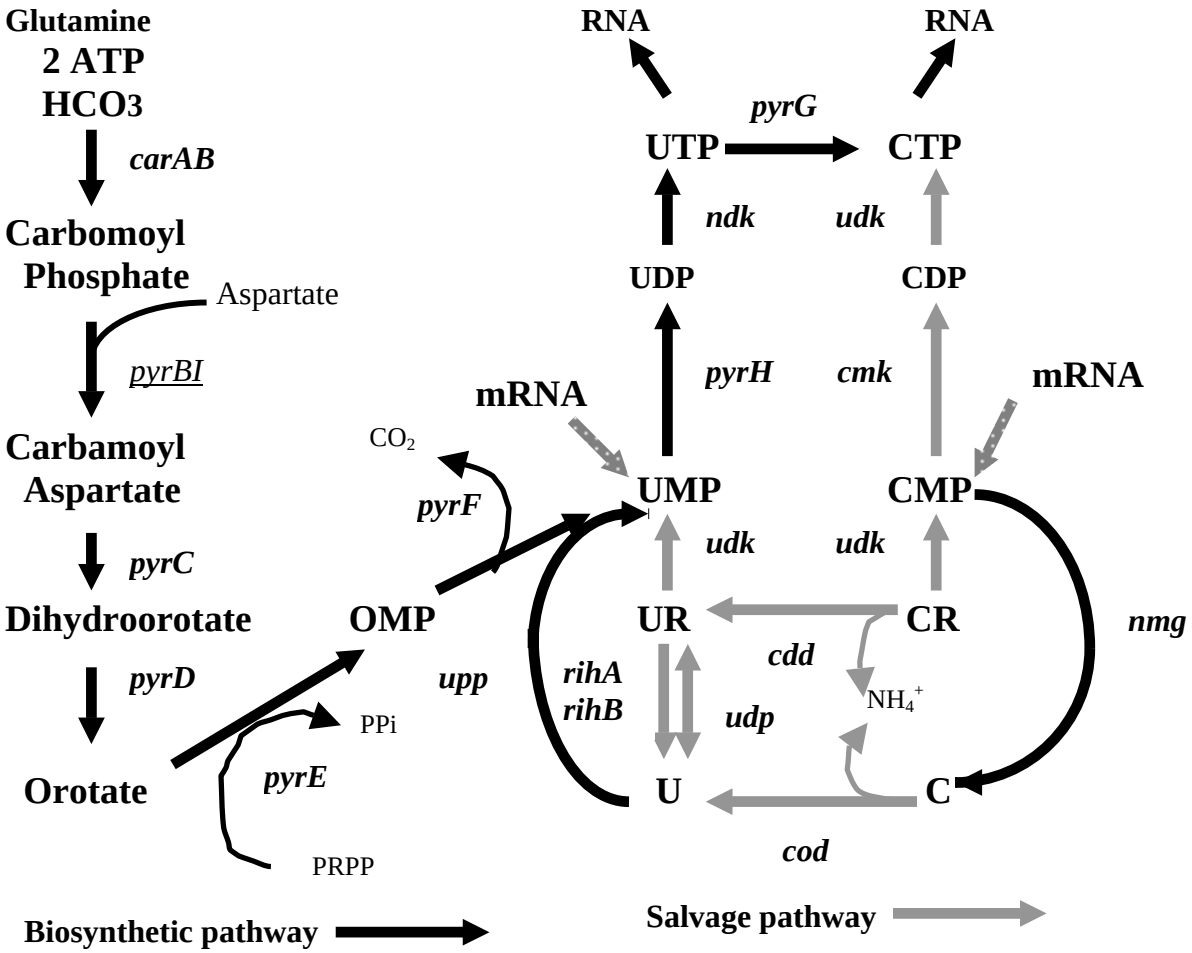
Bordetella pertussis. *Bordetella pertussis* is a Gram-negative, strictly aerobic coccobacillus. *B. pertussis* causes whooping cough in humans, which is marked by its distinct coughing symptom. *B. pertussis* is still a problem today in unvaccinated and under-vaccinated populations. The acellular form of the vaccine has proved to be less than successful (Weiss 1992). Clearly a new and readily acceptable acellular vaccine is needed to control *B. pertussis* infection. This pathogen is difficult to culture outside the host in the laboratory with the result that little is known about its genomic or biochemical properties. What is known is that *B. pertussis* shows a great deal of genetic variation as well as variable genetic expression within single strains (Stibitz, *et al* 1999)

Infection by *B. pertussis* may be divided into three stages, catarrhal, paroxysmal, and convalescence, based on the symptoms exhibited by the subject. The catarrhal stage begins five to 10 days after exposure, and is marked by cold or flu-like symptoms. At this stage isolation of the pathogen is easiest, presumably due to the high concentration of organisms in the airways. This also accounts for the greater contagiousness of the subject at this stage. The paroxysmal stage is most often associated with the *B. pertussis* infection. At this stage the characteristic Whooping" occurs as the infected subject follows a cough with an intake of air through swollen passage ways. During this stage the subject is most vulnerable to secondary infections. The final stage of *B. pertussis* infection is convalescence, and is characterized by a decrease in the acuteness of

symptoms. Once this final stage of the disease is reached, obtaining bacterial cultures of *B. pertussis* from the subject becomes difficult if not impossible. This suggests that the organism has disappeared from the host (Weiss 1992).

The pyrimidine biosynthetic pathway is responsible for the *de novo* synthesis of pyrimidine nucleotides. This pathway is extremely conserved, with little variation in reaction order between organisms (Figures 1-3). The regulation of this pathway in different organisms is by comparison quite diverse. The first committed step in the pyrimidine pathway is the conversion of the precursors carbamoylphosphate and aspartate into carbamoylaspartate and P_i by the enzyme aspartate transcarbamoylase (ATCase) (Figures 1-3). Though ATCase is a highly conserved feature of the pathway, variations have been reported in both its regulation and size. These variations have led to a classification system proposed by Bethell & Jones (1969).

Class A ATCases are dodecameric with a molecular mass of approximately 450-500 kDa (Figure 4). The genes for class A enzymes were originally cloned and sequenced from *Pseudomonas aeruginosa* (Vickrey 1993) and *P. putida* (Schurr 1992 and Schurr *et al.* 1995). Class A genes have more recently been detected in a number of pseudomonads and pseudomonad-like organisms (Kenny *et al.* 1996, Linscott 1996). *P. putida* and *P. aeruginosa* ATCases are dodecamers of six 36.4 kDa *pyrB* polypeptides and six 44.2 kDa *pyrC'* polypeptides. These organisms have a functional ATCase (*pyrB*), nonfunctional DHOase (*pyrC'*) and a functional DHOase (*pyrC*) which catalyzes the next step in the pyrimidine pathway (Schurr *et al.* 1995). More recently, Brichta (2000) and Fields *et al.* (1999) have cloned a second functional DHOase (*pyrC*) from



Biosynthetic pathway genes & enzymes:

carAB: ***Carbamoylphosphate synthetase***
pyrB: ***Aspartate transcarbamoylase***
pyrC: *Dihydroorotase*
pyrD: Dihydroorotate dehydrogenase
pyrE: Orotate phosphoribosyltransferase
pyrG: Cytidine 5'-triphosphate synthetase
pyrH: Uridine 5'-monophosphate kinase
ndk: Nucleotide diphosphokinase

Salvage genes & enzymes:

cdd: Cytidine deaminase
cmk: Cytidine 5'-monophosphate kinase
cod: Cytosine deaminase
nmg: Nucleoside monophosphate glycosylase
upp: Uracil phosphoribosyltransferase
udk: Uridine kinase
udp: Uridine phosphorylase
rihA: Ribonucleoside hydrolase A
rihB: Ribonucleoside hydrolase B

Figure 1. The pyrimidine biosynthetic and salvage pathways in *E. coli*. Reprinted with permission of Seema Patel (Patel 2001).

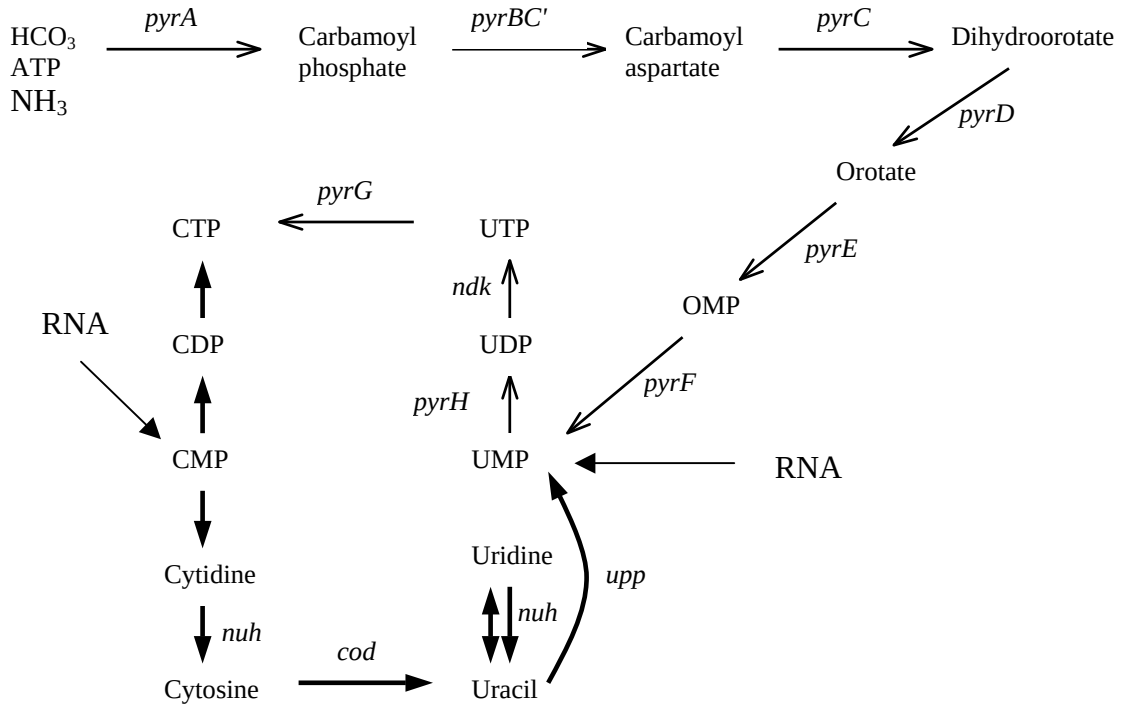


Figure 2. The pyrimidine pathway in *Pseudomonas*. Thin lined arrows outline the pyrimidine biosynthetic pathway. Heavy lined arrows outline the salvage pathway. The enzymes are identified by the following gene designations: *cod*, cytosine deaminase; *pyrA*, carbamoylphosphate synthetase; *pyrB*, aspartate transcarbamoylase; *pyrC*, dihydroorotase *pyrD*, dihydroorotate dehydrogenase; *pyrE*, OMP pyrophosphorylase; *pyrF*, OMP decarboxylase; *pyrG*, CTP synthetase; *pyrH*, UMP kinase; *ndk*, nucleoside diphosphokinase; *nuh*, nucleoside hydrolase; *udp*, uridine phosphorylase, only present in *P. putida*; and *upp*, UMP pyrophosphorylase.
Reprinted with permission of Alan Kumar (Kumar 2000).

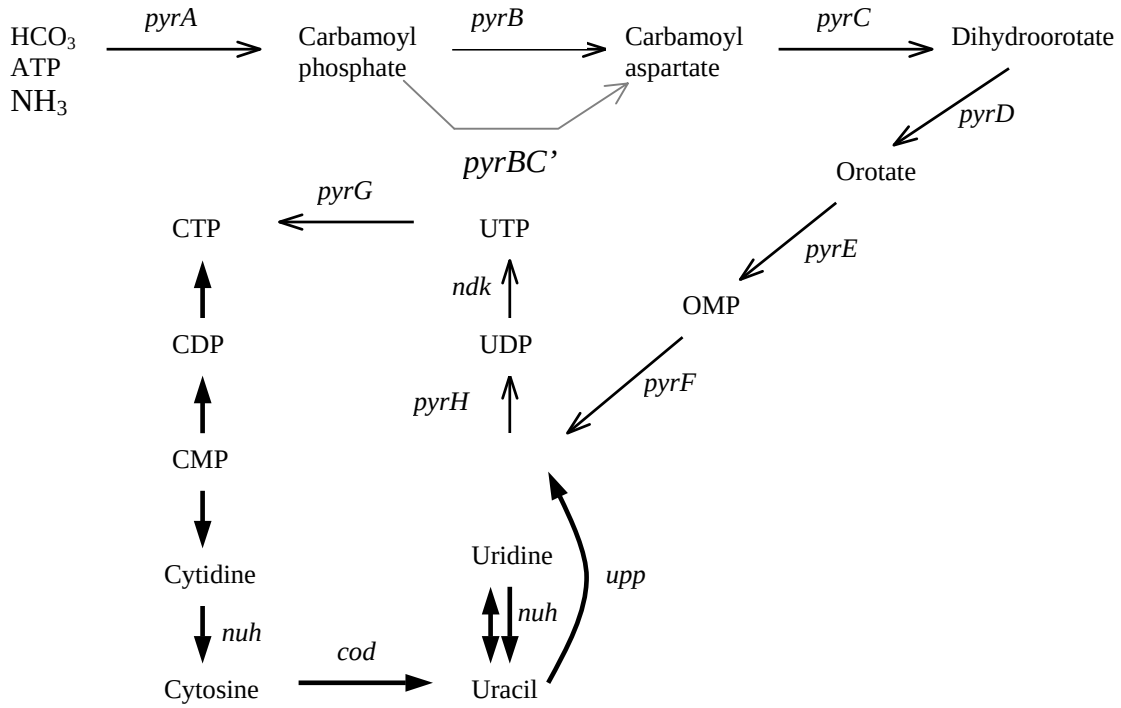


Figure 3. The proposed pyrimidine pathway in *Bordetella pertussis*. Thin lined arrows outline the pyrimidine biosynthetic pathway. Heavy lined arrows outline the salvage pathway. The enzymes are identified by the following gene designations: *cod*, cytosine deaminase; *pyrA*, carbamoylphosphate synthetase; *pyrB*, aspartate transcarbamoylase; *pyrBC'* represents a possible alternative aspartate transcarbamoylase; *pyrC*, dihydroorotase; *pyrD*, dihydroorotate dehydrogenase; *pyrE*, OMP pyrophosphorylase; *pyrF*, OMP decarboxylase; *pyrG*, CTP synthetase; *pyrH*, UMP kinase; *ndk*, nucleoside diphosphokinase; *nuh*, nucleoside hydrolase; and *upp*, UMP pyrophosphorylase.

Adapted and modified version of Alan Kumar's *Pseudomonas* pyrimidine pathway (Kumar 2000).

P. aeruginosa. These DHOases combined, constitute three *pyrC* sequences in this organism. To date, no other organism has been shown to contain more than two *pyrC* sequences.

Class B enzymes are dodecamers like those of class A, but they have a smaller molecular mass. The archetypal organism for class B is *E. coli* with an ATCase of about 310 kDa (Weber 1968). The holoenzyme is formed by six identical *pyrB* polypeptides organized into two trimers and six identical regulatory *pyrI* (DHOase) polypeptides organized into three dimers (Figure 4). This arrangement is the rule among all enteric and class B ATCase bacteria (Wild *et al* 1980)

Class C enzymes are found in all Gram positive bacteria. The enzyme is 100 kDa in size and is typified by that found in *Bacillus subtilis*. The C type enzymes consist of three catalytic polypeptide chains with a molecular mass of 102 kDa (Figure 4). The class C enzymes lack the associated *pyrI* of the class B enzymes (Brabson & Switzer 1975).

Recently the ATCase of *Burkholderia cepacia* was determined to be unlike that of the pseudomonads with which it had previously been classified. It was proposed that the organism be reclassified into a new taxonomic group (Li & West 1995). This led to an investigation of genomic databases to locate organisms that shared homology with the *B. cepacia* ATCase genes. The search results disclosed that *B. pertussis* had a similar ATCase arrangement (ATC1), with a holoenzyme of about 600 kDa exhibits ATCase and DHOase activities. Elsewhere on the *B. pertussis* chromosome, a second ATCase gene (*pyrB*) was found adjacent to a DHOase gene (*pyr C'*), neither of which showed any

expressed activity. The *pyrB* of the *pyrBC' Pseudomonas*-like complex in *B. pertussis* was chosen for investigation. This *pyrB* gene product forms trimers that do not display activity. In all *Pseudomonas* species studied to date, the gene requires the *pyrC'* product to be active. In *B. pertussis* the same complex is seen, but it is not active. I have investigated whether the *pyrB* alone is capable of encoding a functional ATCase. To that end the *pyrB* gene was amplified by PCR and ligated into pT-ADV (Table 1). The resulting recombinant plasmid, pBPATC2 (Table 1) with the *pyrB* gene, was transformed into *E. coli* TOP10F'. The Blue/White screening method described by the pT-ADV manufacturer Clontech, was used. The recombinant plasmid was digested with *Eco*RI and examined by gel electrophoresis. The desired clone was initially selected based on the inserted DNA fragment size (≈ 1.2kbp). DNA sequencing was used for final confirmation. pBPATC2 was then transformed into *E. coli* TB2 pyrB$^-$ and the resultant transconjugants assayed for ATCase activity.

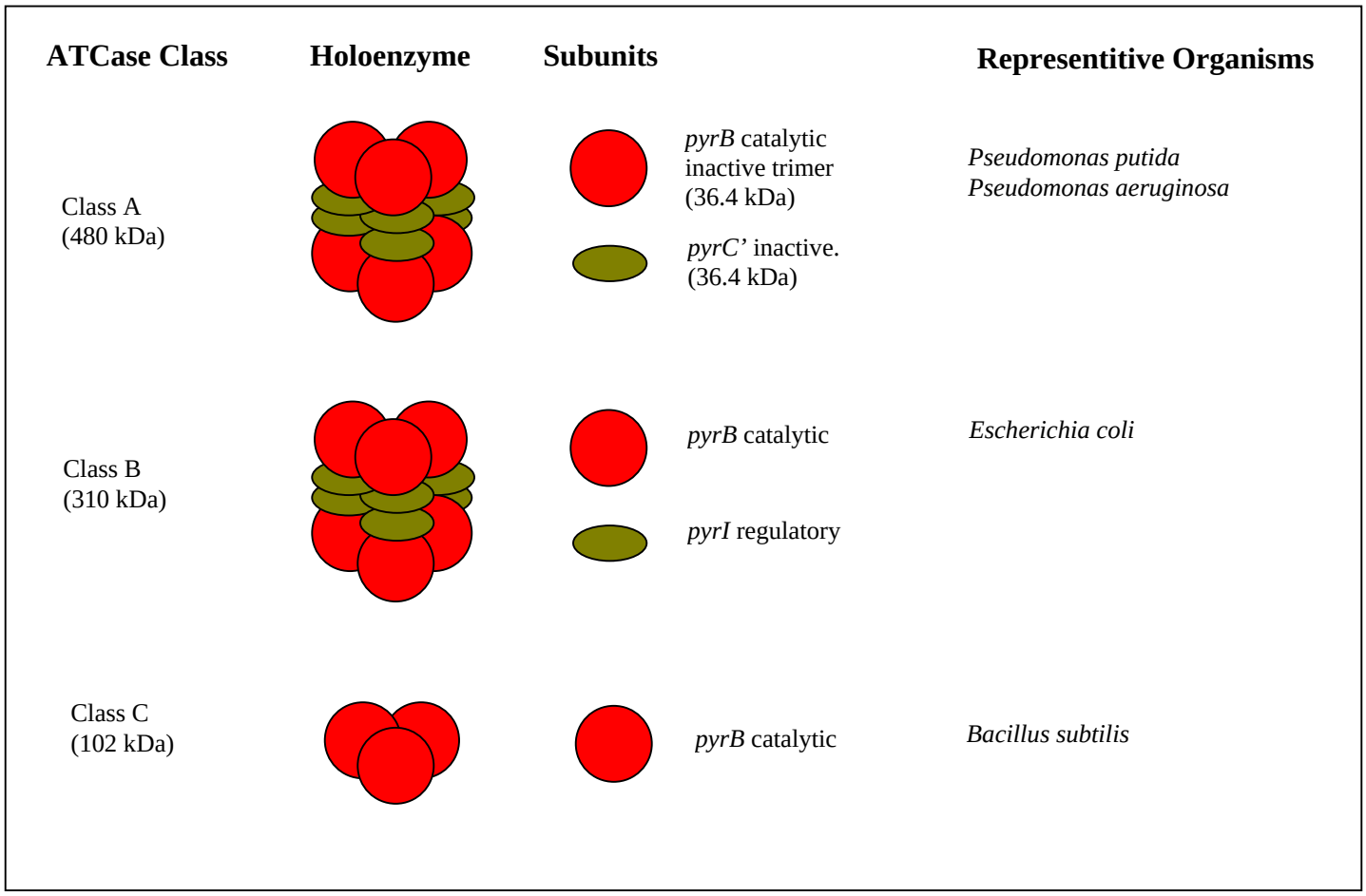


Figure 4. The three ATCase classes

METHODS

Chemicals. Ampicillin monosodium salt, agarose, dibasic potassium phosphate, monobasic potassium phosphate, ammonium sulfate, sodium citrate, magnesium sulfate, thiamine, dextrose, Tris, chloride, ammonium persulfate, sodium dodecyl sulfate, and EDTA disodium salt were all provided by Fisher Scientific Co. Luria-Bertani medium , sodium chloride, potassium chloride, monobasic sodium phosphate, dibasic sodium phosphate, bromophenol blue, charcoal agar, Bordet Gengou agar base, and granulated agar were provided by Difco laboratories. Agarose, low melting point agarose, sarkosyl, aspartate, carbamylphosphate, 5-bromo-4-chloro-3-indoyl-b-D-galactopyranoside (X-gal), isopropyl-B-thio-galactopyranoside (IPTG), proteinase K, bisacrylamide, acrylamide and calcium chloride dihydrate were provided by Sigma Chemical Company. Glycerol, glacial acetic acid, ethanol and sucrose were provided by EM Science. *Bam*HI, *Bam*HI Buffer, *Eco*RI, *Eco*RI Buffer, *Hin*d III, NEB Buffer 2, and 1kb DNA ladder, were all provided by New England Biolabs. PCR was performed using the Advan Taq Plus PCR kit from Clontech Laboratories. Plasmid mini and midi preparation Quantum Prep kits were provided by Bio-Rad.

Type II H_2O was generated by a Barnstead NANOpure ultrapure water system. All Type II H_2O was then autoclaved.

The AdvanTAge© PCR cloning kit was provided by Clontech and included the pT-ADV vector, linearized control DNA template control promoters 1 & 2, 10X buffered

dNTP mix, T4 DNA ligase, 10X ligation buffer, sterile water, bet-mercaptoethanol TOP10F' *E. coli* competent cells, SOC medium, and pUC18.

Bacterial Strains and Plasmids. The bacterial strains and plasmids used in this study are listed in table 1.

Agarose Minigel Electrophoresis. Agarose gel electrophoresis was used to confirm the size as well as presence of DNA throughout this research. A minigel was prepared by using the Fisher Biotech Electrophoresis Midi and Mini-Horizontal system. A 1% agarose gel was made with 1X TAE (diluted 10X TAE 48.4g Tris, 11.4ml glacial acetic acid, 7.5g EDTA, 800ml H_2O, adjusted to pH 8.0), and Fisher Scientific agarose, heated until an even liquid was apparent, and poured into a prepared Fisher Biotech tray with a comb inserted. The gel was allowed to cool and was then submerged in 1X TAE. The comb was then removed. 5X loading dye (25g glycerol, 0.125g bromophenol blue, 0.5ml of 0.5M EDTA, and ddH_2O to 50ml total volume) in 2µl aliquots was mixed by pipeting with 10µl of DNA. 10µl of this sample was loaded into the wells while the gel was submerged. A New England Biolabs 1kb DNA ladder was loaded into the first well, or the center well, depending on the number of DNA samples. Electrophoresis was performed using an EC Apparatus Corporation EC3000P series 90 programmable power unit at 100 volts for 1 hour and 30 minutes or 2 hours and 30 minutes for the larger gel. The gel was later stained in ethidium bromide at 0.5µg/ml and destained by rinsing with water for 5 minutes. Gels were examined using a Bio-Rad Gel Doc 1000 and pictures were printed with a Sony UP-D860 Digital Graphic Printer.

Strain or plasmid	Description of relevant phenotype	Source
Strains		
E. coli		
TOP10F'	Amp^R *lacZ*β	Clontech
TB2	Δ*pyrB*$^-$	Bethesda Research Laboratories, Inc.
Bordetella pertussis		
Tohama I BP348	*bvg*$^-$	Mark Peppler, University of Alberta
Plasmids		
PT-ADV	Amp^R *lacZ*α	Clontech
pBPATC2	Amp^R Δ*lacZ*α *pyrB*	This study

Table 1 *Strains and Plasmids*

Media and Growth Conditions. *E. coli* was grown in Luria-Bertani (LB) broth with aeration and LB agar and minimal medium agar plates at 37°C. Appropriate antibiotics were added to the medium as follows: cells containing the pT-ADV plasmid, ampicillin (100 μg/ml). *B. pertussis* strains were grown in Stainer-Scholte medium and Bordet Gengou agar plates. The *E. coli* minimal medium contained 10.5g K_2HPO_4, 4.5g KH_2PO_4, 1.0g $(NH_4)_2SO_4$, 0.5g Na_3 citrate. These chemicals were dissolved one at a time in 500ml of ddH_2O and autoclaved. After cooling they were combined with a second preparation of 15g agar, 1ml 1M $MgSO_4$, 1ml 1000X thiamine, and 10ml 20%(w/v) glucose, which had also been autoclaved in 500ml of ddH_2O. When growing the *E. coli* pyrimidine auxotroph (TB2), the minimal medium was supplemented with uracil (50μg/ml) and arginine (50μg/ml).

Stainer-Scholte medium was made in two parts (A and B). Part A was prepared as follows: 0.24g L-proline, 0.67g L-glutamic acid, 2.5g NaCl, 0.5g KH_2PO_4, 0.2g KCl, 0.1g $MgCl_2 \cdot 6H_2O$, 0.02g $CaCl_2$, and 6.1g Tris were dissolved in 800ml of ddH_2O and the pH was adjusted to 7.6 using 2.5N HCl. The final volume was adjusted to one liter and the solution was autoclaved for 20 minutes and cooled to room temperature. Part B is a 10X stock solution and was prepared by dissolving 0.4g L-cysteine, 0.2g ascorbic acid, 0.04g nicotinic acid, 1g glutathione, and 0.1g $FeSO_4 \cdot 7H_2O$ in 100ml ddH_2O. This solution was then filter sterilized. Part B was added to part A at ratio of 1:100. 20% sterile casamino acids were added as growth enhancer after parts A and B were combined (Stainer 1970, Imaizumi *et al.* 1983).

Bordet Gengou/10% rabbit blood agar plates was made by adding 30g of Bordet Gengou Agar Base with 1 liter of ddH_2O with 10g of glycerol. The ingredients were heated to boiling to suspend the contents completely. This mixture was sterilized in the autoclave and cooled to 45°C before rabbit blood was added to make the final concentration 10%.

Chromosomal isolation. An isolated colony of *B. pertussis* Tohama BP348 was taken form a 48 hour growth on Bordet Gengou/10% rabbit blood agar plate and emulsified in 2mls of Stainer Sholte medium. 100µl aliquots were spread on 15 plates of Bordet Gengou/10% rabbit blood plates. These plates were incubated for 48 hours at 35°C. The resulting lawn growth was scraped from the agar plates with a sterile glass slide and placed in a sterile disposable 50ml Falcon® tube with 26ml of Tris-buffered saline (TBS) and vortexed to evenly suspend the cells. The cells were centrifuged for 5 minutes at 4,000xg at 4°C and washed with 25mls of TBS and centrifuged once more. The wash was then repeated. The resulting pellet was then suspended in 2.5 ml of TBS and placed in a 37°C waterbath. 2.5ml of 1% sarkosyl/100 mM EDTa pH8.0 was preheated to 50°C and added and the 5ml mixture was placed in a 50°C waterbath. 500µl of a 20mg/ml proteinase K solution were added and left overnight for digestion. The digested protein was removed by repeated extractions with equal volumes of chloroform/isoamyl alcohol (24:1) until all precipitated proteins were removed. One final protein extraction was affected by using an equal volume of buffered phenol pH 8.0, chloroform, isoamyl alcohol (25:24:1). Residual traces of organic solvents were removed by extraction with an equal volume of diethyl ether. Two and one half volumes of cold

100% ethanol (-20°C) were slowly added to create two phases. The genomic DNA was collected by spooling onto a glass rod. The spooled DNA was rinsed in 70% ethanol and drained well. The genomic DNA was then dissolved in 5ml of 1 X TE buffer. 5µl of a 10mg/ml solution of RNase was then added. The DNA was then examined on a 1% agarose gel to determine size and quantity.

Polymerase chain reaction. Amplification of the *pyrB* gene from *B. pertussis* was achieved with polymerase chain reaction (PCR). The primers were constructed by Intergrated DNA Technologies to amplify the pyrB gene. The PCR mixture contained 1µl of isolated chromosomal DNA, 80ng of forward primer, 80ng of reverse primer, 5µl of 10x PCR buffer, 2µl 100 mM dNTPs, 2µl of *Taq* polymerase, and sterile ddH_2O to bring the mixture up to 50µl. The cycling for the PCR were: 94°C for 3 minutes, 30 cycles of 94 °C for 30 seconds and 65°C for 30 seconds, 72°C for 5 minutes, and hold at 4°C. DNA was checked on 1% agarose gel for size and quantity.

PCR product and vector ligation preparation. The PCR product was prepared for ligation by placing 20ul of the product into a 0.5ml microfuge tube with 2µl of 3M Na Acetate and 66µl of 100% ethanol. The contents were mixed by pipetting and placed on dry ice for 15 min. After 15 minutes the tube was centrifuged at 4°C at 6,702xg for 10 minutes. The ethanol was removed and 1ml of 70% ethanol was added. The tube was inverted once to mix and placed on dry ice for 5 minutes. The tube was then spun at 4°C at 6,702xg for 5 minutes. The supernatant was drawn off and the tube and its contents

were placed in the speedvac® for 5 minutes until dry. The pellet was then resuspended in 10µl of ddH_2O and the contents were analyzed on an agarose gel for confirmation.

The pT-ADV vector was digested with *Eco*RI. After the digest 1µl of alkaline phosphatase was added. The vector and alkaline phosphatase was incubated at 37°C in a water bath for 1 hour. After that time 10µl of ddH_2O and 2µl of 3M Na acetate + 66µl of 100% ethanol was added. The contents were mixed by pipetting and placed on dry ice for 15 minutes. After 15 minutes the tube was centrifuged at 4°C at 6,702xg for 10 minutes. The ethanol was removed and 1ml of 70% ethanol was added. The tube was inverted once to mix and placed on dry ice for 5 minutes. The tube was then spun at 4°C at 6,702xg rpm for 5 minutes. The supernatant was drawn off and the tube and its contents were placed in the Speedvac® for 5 minutes until dry. The pellet was then resuspended in 10µl of ddH_2O and the contents were analyzed on an agarose gel for confirmation.

PCR product and vector ligation. In a 0.5ml microfuge tube 1µl of 1/5 dilution of prepared pT-ADV, 4µl of PCR product, 1µl of 10 X T4 ligase buffer, 3µl of ddH_2O, and 1µl of T4 ligase were combined and incubated in a 12°C cooler for 24 hours.

Chemical transformations ($CaCl_2$). *E. coli* cells were inoculated into 50ml of LB and grown to mid-log. The cells were then put on ice for 10 minutes and centrifuged for at 4°C for 5 minutes at 7,300xg. The supernatant was decanted, and the cells were washed with 50mM $CaCl_2$ and 10% glycerol. This step was repeated twice more with the

final pellet being resuspended in 2ml of $CaCl_2$ and 10% glycerol. The cells were chilled at 4°C for 16 hours, then divided into 200µl portions and stored at -80°C for future use.

The transformation was carried out by allowing 100µl of $CaCl_2$ competent *E. coli* cells to interact on ice for 30 minutes with 3µl of plasmid DNA. The cells and plasmid were then heat-shocked for 50 seconds at 42°C in a water bath. The mixture was subjected to a cold shock on ice for 2 minutes. 500µl of LB were added to the transformed cells, which were then plated in 50µl amounts on LB/amp^{100}, IPTG, and X-gal and grown overnight at 37°C in a Fisher Scientific IsoTemp incubator. Clones were screened using the blue/white method. The white colonies indicating the interruption of the *lacZ*α gene, by the successful inserted PCR product. Patch plates were made from the resulting white colonies and grown overnight as before, and then stored at 4°C overnight to verify color.

Plasmid peparation of clones. For initial screening a modified version of the BioRad plasmid miniprep kit was used, making use of the reagents provided in the kit. Patch plates were removed from the 4°C refrigerator and colonies displaying white phenotype were selected for inoculation in 5ml of LB/amp^{100} broth and incubated overnight at 37ºµl with aeration. The tubes of overnight growth were then removed and treated as follows. An aliquot of 1.5ml of the overnight culture was centrifuged 30 seconds at 13,196xg to pellet the cells. The supernatant was pipeted off and 200µl of BioRad prep kit suspension solution was added and the pellet resuspended. 250µl of BioRad lysis solution was then added and mixed by inversion, followed by 250µl of

neutralizing solution mixed by inversion. The chromosomal DNA and cellular debris were then centrifuged at 13,196xg for 10 minutes to pellet. The supernatant was collected and the resulting pellet discarded. 700µl of 100% isopropanol was added to the supernatant which was then mixed by inversion and allowed to interact for 5 minutes to precipitate the plasmid.. Next, the sample was centrifuged for 15 minutes at high speed. The supernatant was removed and pellet was washed by 5 minute centrifugation with 1ml of ice-cold 70% ethanol. Ethanol was drained off and pellet was allowed to dry in a 60ºC dry incubator. 50µl of 1X TE buffer was added and plasmid was stored in -40º freezer. Gel electrophoresis was run on samples (see procedure) using a 1kb marker provided by New England Biolabs, and stained with ethidium bromide. Subsequent purification was done using the methods described by Bio-Rad in its midi and miniprep kits.

Nuclease digestions. Digestions were performed in 20µl volumes consisting of 5µl plasmid sample, 2µl of 10X restriction buffer, 1µl of restriction enzyme, 12µl of ddH_2O. All ingredients were combined on ice except the plasmid sample. The plasmid was added last, just prior to placing in the 37º water bath for approximately one hour.

Aspartate transcarbamoylase assays. For the ATCase assay 20ml cell cultures were centrifuged during late log phase and washed and resuspended in 5ml of ATCase buffer containing 5ml 1M Tris pH 8.0, 200µl 1M beta mercaptoehanol, and 2µl 1M $ZnSO_4$ brought to 100ml with ddH_2O. This suspension was then subjected to sonication at 4ºC for 4 min. The following reagents were mixed in the following amounts: 546µl ddH_2O, 28µl tri-buffer (50mM MES, 100mM diethanolamine, and 51mM N-ethylmorpholine, pH 9.5), and 35µl 20X aspartate (200mM aspartate). 87µl of this

mixture was distributed into each of 6 microtiter wells. To these wells was added 3µl of sample enzyme obtained by sonication. The reaction was initiated by adding 10µl of carbamoylphosphate to each well. The samples were incubated 20 minutes at 30ºC (Adair & Jones 1972). After incubating, the reaction was stopped by adding 100µl of stop mix (2 parts antipyrine and 1 part monoxime, combined just before use). The samples were incubated in the presence of light for a further 2 hours at 60ºC (Prescott & Jones 1969). The samples were then read on the Molecular Devices Company microplate reader.

Non-denatureing (native) Polyacrylamide gel electrophoresis(PAGE). To test for ATCase activity a native PAGE was run. The separating gel was prepared by degassing a mixture containing 6ml of 30% acrylamide/0.8% bisacrylamide, 3.75ml of 4X Tris-Cl/SDS pH 8.8 (91g Tris base, 300ml ddH_2O, pH adjusted with 1N HCl and brought to 500ml with ddH_2O), 5.25ml of ddH_2O for 5 minutes. After degassing 0.05ml of 10% ammonium persulfate and 0.01ml of TEMED were added and swirled to mix. The PAGE apparatus was assembled and the separating gel solution was added to the apparatus with a Pasteur pipet to a height of 11cm.

Another Pasteur pipet was used to cover the top of the gel with a 1cm layer of H_2O-saturated isobutyl alcohol. Gel was allowed to polymerize for one hour at room temperature. While the gel was polymerizing a stacking gel was made by combining 0.65ml of 30% acrylamide/0.8% bisacrylamide, 1.25ml of 4X Tris-Cl pH 6.8, and 3.05ml ddH_2O. The mixture was degassed for 15 minutes and 25µl of 10% ammonium

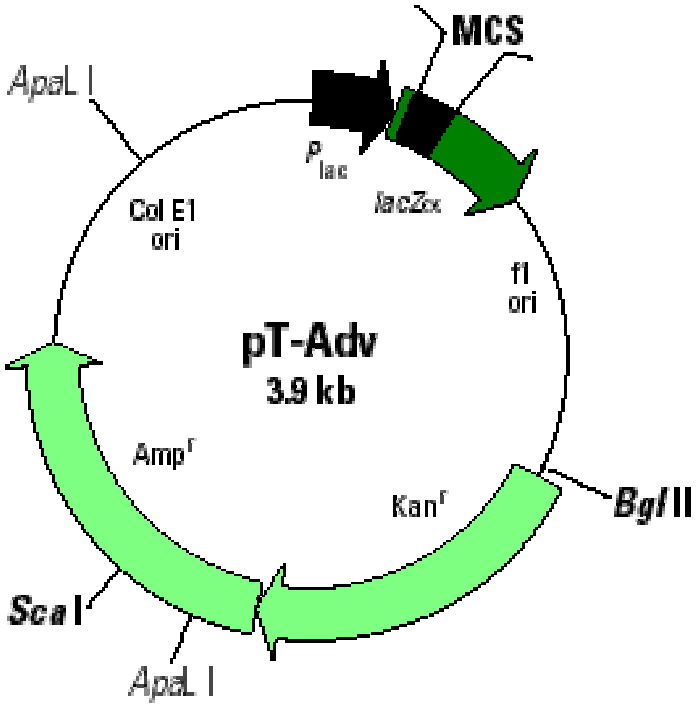
persulfate and 5µl of TEMED were added. The solution was mixed by swirling. The alcohol was then poured off of the gel and the top was rinsed with 1Xtris-Cl, PH 8.8. The stacking gel solution was then added and a comb inserted. The gel was allowed to polymerize for 45 minutes.

After polymerization, a 1:1 dilution of the sample protein and 2X sample buffer was heated for 3 minutes at 100°C in a sealed microcentrifuge tube. The comb was removed from the gel and the wells were rinsed with 1X electrophoresis buffer and then filled with the same buffer. The sample was then loaded into the wells, as were the standards. 1X electrophoresis buffer was then used to fill in all the empty wells and to fill the chamber to the upper electrode.

The chamber was sealed and connected to the power supply and run at 100 volts until the bromphenol blue tracking dye reached the bottom of the separating gel (approximately 1 hour). The gel was then stained with Coomasie blue.

RESULTS

Cloning of Bordetella pertussis *pyrB*. To test the *Pseudomonas*-like aspartate transcarbamoylase gene from *B. pertussis* it was necessary to create a clone from a PCR product. This product was the gene encoding the catalytic subunit *pyrB*. The gene sequence was ligated onto the pT-ADV at the *Eco*RI site (Figure 5). This new plasmid (pBPATC2) was transformed into beta-mercaptoethanol competent *E. coli* TOP10F' cells and plated on LB with ampicillin at 100µg/ml, IPTG and X-gal (LB amp^{100} X-gal) for blue/white screening. The resulting white colonies were patch-plated on LB amp^{100} X-gal plates and allowed to grow overnight at 37°C. Multiple patches were selected for plasmid isolation. pBPATC2 was compared by electrophoresis on a 1% agarose gel (Figure 6). Patches number 16 and 17 revealed bands around , 5.6 kb (the approximate size of the expected clone). pBPATC2 was cut with *Eco*RI using a 20µl digestion and run on a 1% agarose gel for comparison (Figure 7). Number 17 revealed two bands; one around 5 kb (the approximate size of the vector) and one around 1.2kb (expected size of the *pyrB* gene). A sample of clone 17 was sent to Lone Star Labs, Inc in Houston, Texas confirming that the cloned DNA fragment was indeed the *B. pertussis pyrB* gene and in the correct orientation.



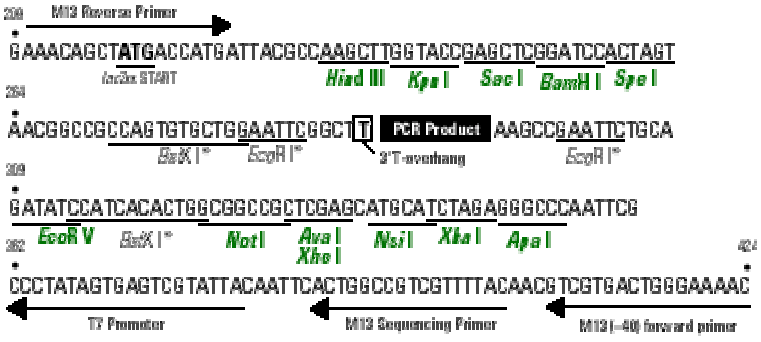


Figure 5. pT-ADV and the multiple cloning site. Clontech 1999.

ATCase assay of the *B. pertussis* clone. The pBATC2 plasmid was transformed into *E. coli* TB2 *pyrB*$^{-}$ and the modified ATCase Assay (see ATCase assay methods) was run. The assay revealed no ATCase activity. A native activity gel was performed to confirm these findings. A second ATCase assay was run with pBATC2 transformed into *E. coli* TB2 *pyrB*$^{-}$ *pyrC*$^{+}$. The expected ATCase activity was not detected in this second assay.

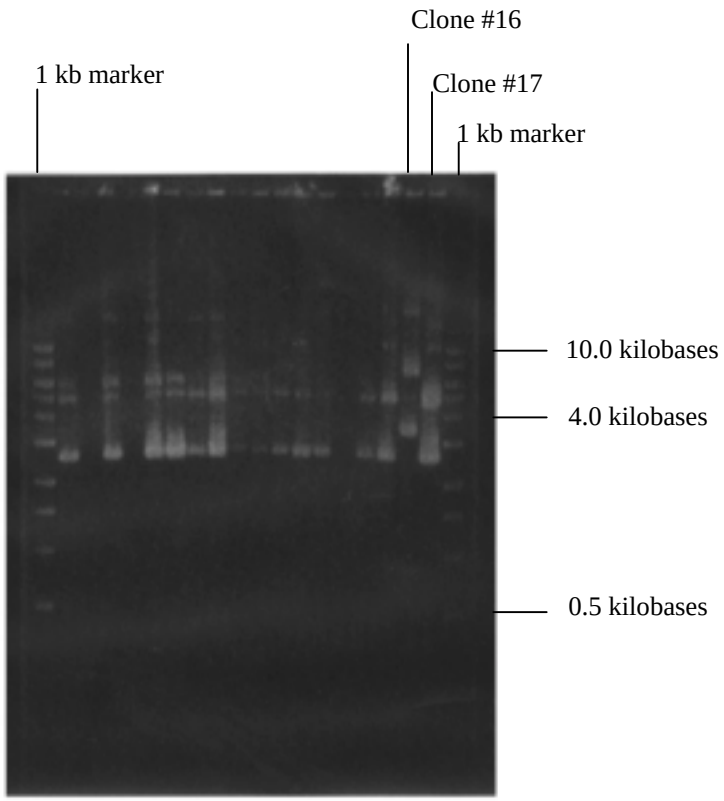


Figure 6. Plasmid preparation of clones 1-17, taken from patchplates.

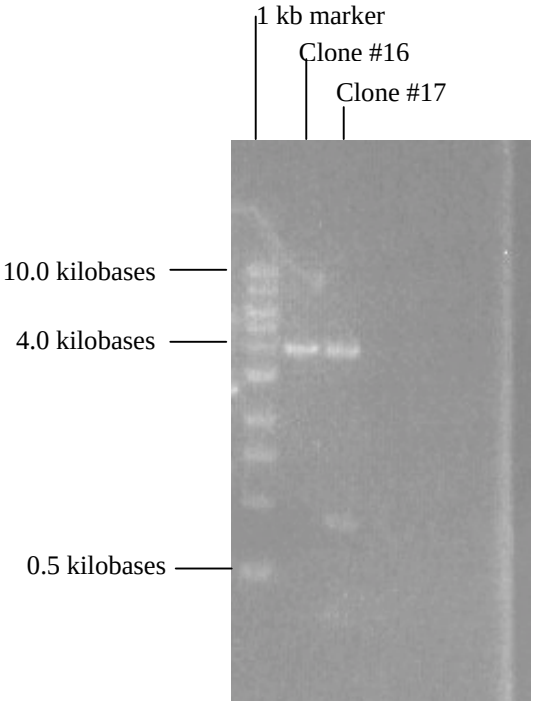


Figure 7. *Eco*R1 digestion of clones 16 & 17. 17 exhibited a second band around 1kb.

DISCUSSION

The *Bordetella pertussis* genome has been sequenced and contains 4,086,186bp. The *pyrBC'* complex can be found at 324,479bp (Figure 8) with the *pyrB* portion amounting to only 927bp compared to the 1065bp originally suggested. The difference is explained by a misidentified start codon. The proper start codon is 138bp downstream of the earlier misidentified start codon. However, no appropiate ribosomal binding site was found in the vicinity of the *pyrB* gene. The adjacent *pyrC'* has a premature stop that appears to be a nonsense mutation. When the same region is compared with that of *Bordetella bronchiseptica* and *Bordetella parapertussis*, the sequence TGA is no longer a stop, but is instead TTG. If indeed the *pyrC'* polypeptide performs a scaffolding role in the holoenzyme with the *pyrB* adjacent to it, then the premature stop would likely render this gene faulty.

The *pyrBC'* complex is part of a larger operon containing four additional genes. These genes were found in the section of the operon examined. The examined sequence amounted to 3910bp, and neither the beginning nor the end of the operon lay withing this sequence. Thus, it is likely that more genes lie within this operon. It should also be noted that a search of the genome database revealed no gene that could be identified as *pyrR*.. Overlapping the *pyrC'* gene after the early stop is the fourth gene seen in this operon, 1-acyl-sn-glycerol-3-phosphate acyltransferase (AGPAT). *B. bronchiseptica*

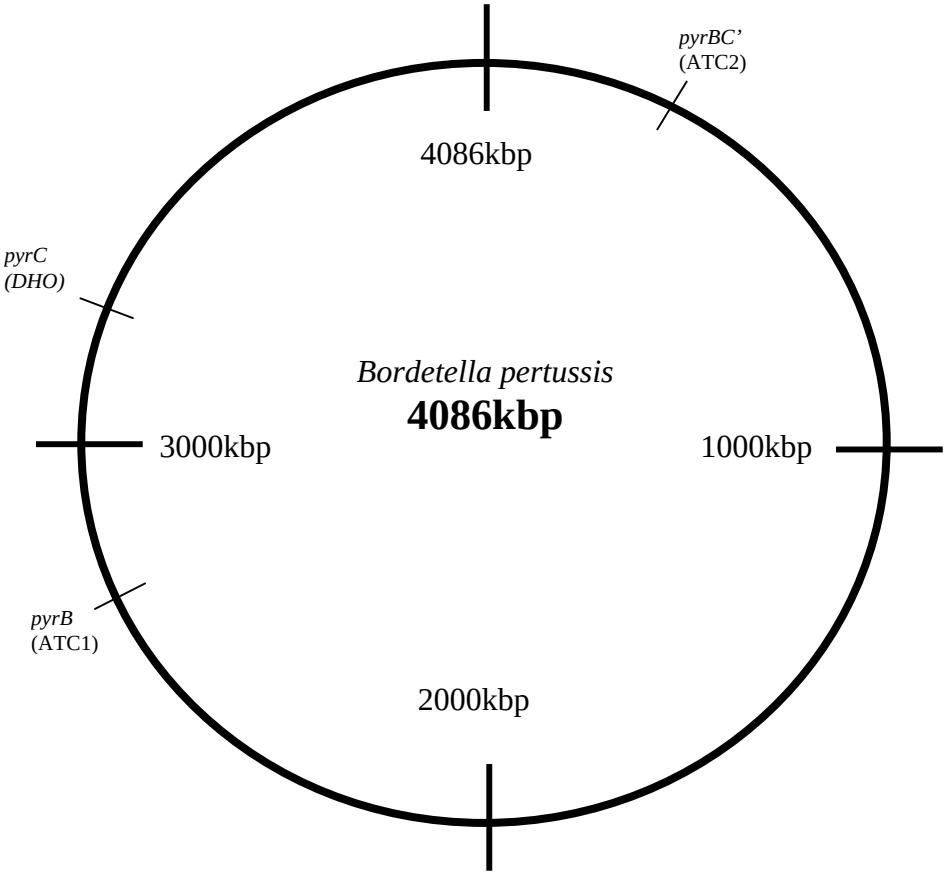


Figure 8. The *Bordetella pertussis* chromosome and the *pyrB*, *pyrBC'*, and *pyrC* genes.

and *B. parapertussis* were not examined to determine if they held the same overlapping sequence.

The presence of the *pyrBC'* complex in *B. pertussis, B. bronchiseptica,* and *B. parapertussis* suggests that the complex existed in a common ancestor. The premature stop mutation found only in *B. pertussis*, shows that the mutation occurred sometime after the ancestral split. The fact that the gene was conserved while the ancestor evolved into three distinct species, indicates that the gene is still fulfilling some function that the organism finds favorable. How long the *pyrBC'* complex with the nonsense mutation is maintained in *B. pertussis* may provide evidence as to whether this mutant gene still provides a beneficial product or is now a lost gene.

The pBPATC2 failed to complement the *pyrB*$^{-}$ *E. coli* TB2 and produce any ATCase activity. This was confirmed by both an ATCase assay and a native PAGE activity gel. It is not know whether this lack of activity is due to the possibility that no protein was made, that an inactive trimer was produced, or that inactive monomers were produced. Co-transformation with a plasmid containing *pyrC'* from *Pseudomonas aeruginosa* was an afterthought, and should not dissuade one from the idea that the *pyrC'* from *B. pertussis* might yet provide the key to the expression of the second ATCase in *B. pertussis*. The premature stop in the *pyrC'* portion of the complex, however, does not make this a promising pursuit.

The logical next step should be the cloning of *pyrC'* from *B. bronchiseptica* or *B. parapertussis*. The presence of the *pyrBC'* complex in these two closely related organisms, and the fact that the premature stop is not present in either of their sequences

make them the obvious choice for *pyrBC'* expression in a construct containing the second *pyrB* from *B. pertussis*.

A second avenue of exploration is already underway. Master student Emily Carey has been working with the first *pyrB* gene in *B. pertussis* (ATC1). She has cloned this gene, and is attempting to make a knockout of that gene for reintroduction into the *B. pertussis* chromosome by homologous recombination. A wild type *B. pertussis* with this knockout inserted may provide a hint as to the purpose of what seems to be a disabled gene.

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